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Zebrafish Serotonin-*N*-Acetyltransferase-2 Gene Regulation: Pineal-Restrictive Downstream Module Contains a Functional E-Box and Three Photoreceptor Conserved Elements

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Pineal function is defined by a set of very narrowly expressed genes that encode proteins required for photoperiodic transduction and rhythmic melatonin secretion. One of these proteins is serotonin *N*-acetyltransferase (arylalkylamine *N*-acetyltransferase, AANAT), which controls the daily rhythm in melatonin production. Here, pineal-specific expression of the zebrafish *aanat-2* (*zfaanat-2*) was studied using *in vivo* transient expression analyses of promoter-reporter constructs; this revealed that specificity is determined by two regions located 12 kb away from each other. One is the 5′-flanking region, and the other is a 257-bp sequence, located 6 kb downstream of the transcribed region. This 3′-sequence, designated pineal-restrictive downstream module (PRDM), has a dual function: enhancement of pineal expression and inhibition of extrapineal expression. The former is an autonomic property of PRDM whereas the later function requires interaction with the upstream regulatory region of *zfaanat-2*. Functional analyses of the PRDM sequence revealed that three photoreceptor conserved elements (TAATC) and a single perfect E-box (CACGTG) are crucial for the dual function of PRDM. These results indicate that pineal specificity of *zfaanat-2* is determined by the dual functionality of the PRDM and the interaction between upstream regulatory region and downstream photoreceptor conserved elements and E-box element. (*Molecular Endocrinology* 18: 1210–1221, 2004)

THE PRIMARY ROLE of the pineal gland (epiphysis) is to transduce photoperiodic information into daily and annual physiological changes through rhythmic production and secretion of melatonin; in all vertebrates, high circulating levels of melatonin occur at night (1). A secondary site of melatonin production is the retina photoreceptor layer where melatonin is thought to play a paracrine role in visual adaptation to darkness (2). In essentially all vertebrates, a circadian clock drives the rhythmic production of melatonin. In mammals, the clock controlling the pineal gland is located in the hypothalamic suprachiasmatic nucleus, whereas rhythmic melatonin production in retinal photoreceptors is controlled by an intrinsic circadian oscillator (3). In lower vertebrates, the pineal gland, as well as the retina, is photoreceptive and contains an intrinsic circadian pacemaker that drives the rhythmic production of the melatonin signal (4). These features require the expression of an array of specific genes in the pineal gland that are involved in photoreception, phototransduction, clock function, and melatonin production.

The melatonin rhythm is generated by changes in the activity of arylalkylamine *N*-acetyltransferase (AANAT), the key enzyme in the melatonin production pathway. The increased production of melatonin during the night reflects increased AANAT activity, and termination of melatonin production by light is due to proteasomal degradation of the enzyme (5–8). In addition, in several species, *aanat* transcription exhibits a robust circadian rhythm that is regulated by a circadian oscillator (9–13). In the chicken pineal gland (14) and the rat retina (15), this rhythm is mediated through an E-box regulatory site, which mediates rhythmic expression of clock-controlled genes (16, 17). The

Abbreviations: AANAT, Arylalkylamine *N*-acetyltransferase, serotonin *N*-acetyltransferase; CMV, cytomegalovirus; CRX, cone rod homeobox; DsRed, *Discosoma* red fluorescent protein; EGFP, enhanced green fluorescent protein; OTX, orthodenticle homeobox; PCE, photoreceptor conserved element; PIRE, pineal regulatory element; PRDM, pineal-restrictive downstream module; *zfaanat 2*, zebrafish AANAT; zOTX-5, zebrafish OTX-5.

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presence of E-box elements in the 5′-regulatory region of several fish *aanat*s, including that of the zebrafish, suggests that rhythmic expression of this gene in fish may also be controlled by this element (18). Accordingly, vertebrate *aanat* is recognized as a clock-controlled gene that serves as a link between the circadian clock and the output signal—melatonin.

Fish contain two *aanat*s, *aanat-1* and *aanat-2*, which are preferentially expressed in the retina and pineal gland, respectively (19, 20). Developmental studies in the zebrafish (zf, *Danio rerio*) indicate that pineal *zfaanat-2* expression begins at 22 h post fertilization, and a circadian clock-controlled rhythm of its transcript begins 2 d post fertilization (13, 21). This expression pattern, together with the advances in zebrafish circadian system research (22, 23), makes *zfaanat-2* an attractive model for studying the molecular mechanism of tissue specificity in the clock-containing pineal gland.

Recently, transgenic zebrafish lines, TG(AANAT2:EGFP), have been established in which enhanced green fluorescent protein (EGFP) is expressed in the pineal gland under control of the *zfaanat-2* regulatory regions (18). Pineal specificity in these lines is dependent on a 1.6-kb upstream region and a 3.5-kb downstream sequence. In the present study, pineal specificity of *zfaanat-2* was found to be conferred by a downstream cluster of functional *cis*-acting regulatory elements located within a 257-bp sequence termed PRDM (pineal-restrictive downstream module).

RESULTS

Identification of the Downstream Regulatory Region that Drives Pineal-Specific Expression of *zfaanat-2*

The portion of the 3.5-kb downstream sequence that determines tissue specificity was identified using *in vivo* transient expression of promoter-reporter constructs. Constructs contained the *zfaanat-2* promoter, EGFP reporter gene, and fragments of the downstream 3.5-kb sequence.

Injection of the construct, which contained the entire 3.5-kb downstream sequence (AANAT2-EGFP-3.5 kb, Fig. 1A), resulted in EGFP expression in 35% of injected embryos. Among these EGFP-positive embryos, a signal in the pineal gland (Fig. 1, E–H) was observed in 88% (Fig. 1A). In half of these embryos the signal was restricted to the pineal gland (Fig. 1, G and H); the other half exhibited a low level of ectopic EGFP expression as well (Fig. 1, E and F). Ectopic expression of EGFP alone was observed in only 12% of EGFP-positive embryos (Fig. 1A). In contrast, injection of the construct that lacks the 3.5-kb sequence (AANAT2-EGFP, Fig. 1A) resulted in ectopic expression only, with no signal in the pineal gland (Fig. 1, C and D). These results confirm that the 3.5-kb downstream sequence contains regulatory elements that confer pineal-specific expression, corroborating published results obtained with a different vector (18).

The region within the 3.5-kb downstream sequence of *zfaanat-2* sufficient to direct pineal-specific gene expression was determined using a set of eight serial constructs, each of which contained a partial but overlapping fragment derived from this sequence (AANAT2-EGFP-a through -h, Fig. 1A; see *Materials and Methods*). All embryos injected with constructs AANAT2-EGFP-a, -b, -c, -f, -g, and -h that were EGFP positive showed low levels of ectopic expression and no pineal EGFP expression (Fig. 1A). In contrast, embryos injected with constructs AANAT2-EGFP-d and AANAT2-EGFP-e exhibited pineal EGFP expression (96% and 91% of EGFP-positive embryos, respectively). In addition, the proportion of embryos exhibiting ectopic expression was reduced ($P < 0.001$) in these injections. This indicates that the overlapping fragments D and E contain important regulatory elements. Because most of fragment D is included in fragment E (Fig. 1A) and fragment D alone was sufficient to induce pineal-specific expression, it was further investigated.

The overlapping region, AANAT2-EGFP-d1 (Fig. 1A), generated a significantly ($P < 0.01$) reduced level of pineal specificity and higher ectopicity as compared with fragment D: of the 18% EGFP-positive embryos, 62% had an EGFP signal in the pineal gland, and the remaining exhibited only ectopic expression (Fig. 1A). This finding, compared with the 96% pineal expression obtained with AANAT2-EGFP-d, suggests that elements present in the 5′-end of fragment D, not included in D1, contribute to the activity of this fragment. This was tested using a construct containing 95 bp from the 5′-end of fragment D, AANAT2-EGFP-d2. Most (96%) of the EGFP-positive embryos injected with this construct exhibited ectopic expression only, and pineal expression was significantly reduced ($P < 0.001$; Fig. 1A).

Among the fragments that significantly ($P < 0.001$) induced pineal expression, D, D1, E, and the entire 3.5-kb fragments D and E generated a significantly ($P < 0.01$) higher proportion of pineal-expressing embryos. In addition, fragment D generated a significantly ($P < 0.01$) lower proportion of ectopic expressing embryos. This indicates that the 257-bp of fragment D contains multiple interacting elements that influence pineal-specific expression.

Similar results were obtained in a subsequent experiment using a different construct, AANAT2-*Discosoma* red fluorescent protein (DsRed)-d; in these studies, ectopic expression was monitored by coinjection of AANAT2-EGFP. This use of an internal reference normalizes for possible variations in injection efficiencies and mosaic expression, thereby eliminating artifacts due to embryo-to-embryo variations. In these coinjection experiments, 90% of the DsRed-positive embryos exhibited pineal-specific expression (Fig. 1, B and I) whereas among the EGFP-positive embryos, 100% were ectopic (Fig. 1B). Most importantly, more

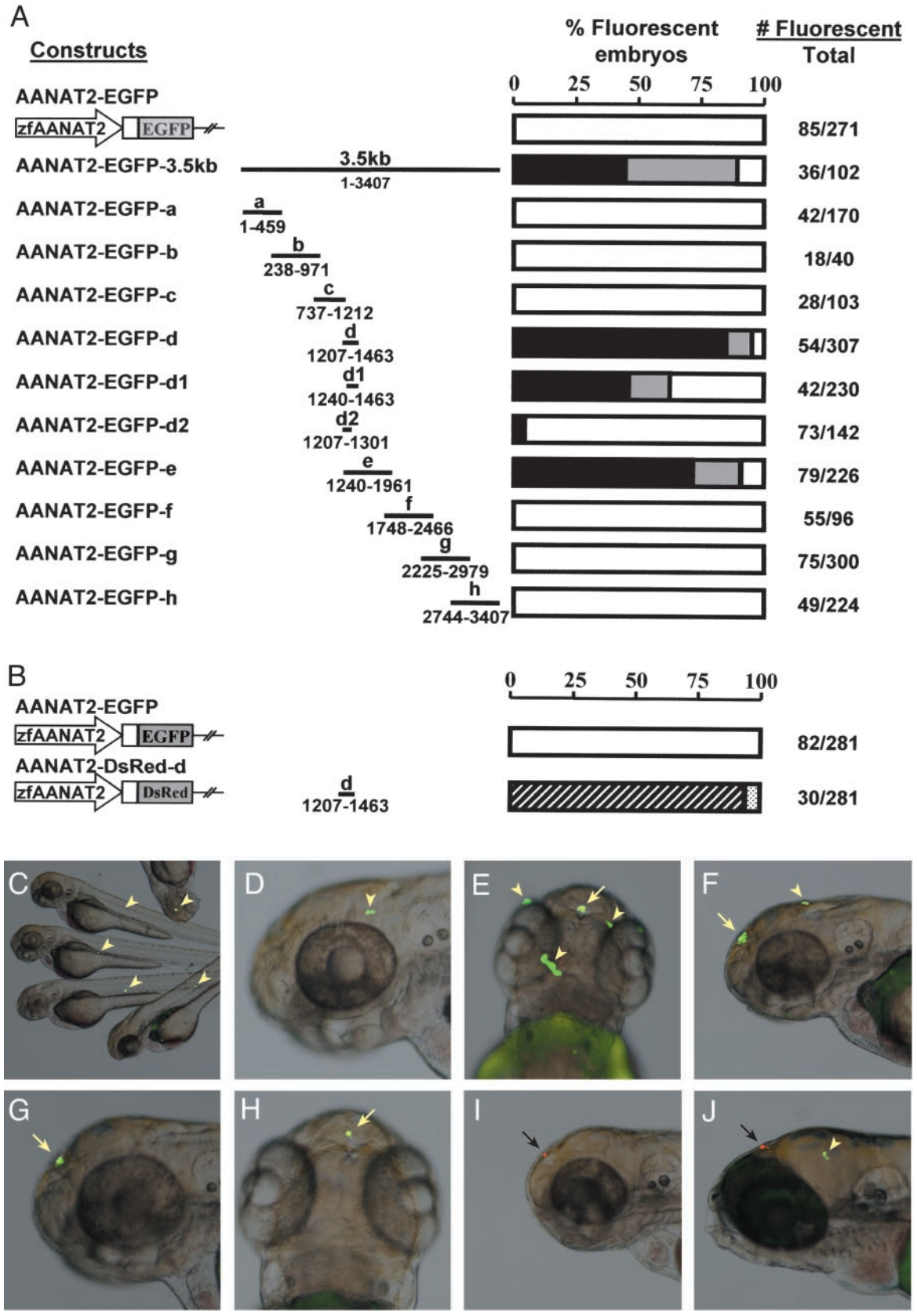

Fig. 1. Isolation of PRDM

A, The 3.5-kb downstream sequence and derived fragments were inserted in the AANAT2-EGFP vector (schematically drawn at the *top*). The names of the resulting constructs are shown on the *left*. The relative position of the tested fragments within the

than half of the pineal gland-specific DsRed-expressing embryos also showed ectopic EGFP expression (Fig. 1J). These results confirm those of the single injection experiments and validate the use of a transient expression assay approach to identify *zfaanat-2* regulatory regions.

These experiments make it clear that fragment D plays an essential role in enhancing pineal-specific expression of *zfaanat-2*. The apparent specificity conferred by fragment D has two components. One is the enhancement of pineal expression, and the other is suppression of extrapineal expression. Accordingly, this fragment is designated pineal-restrictive downstream module (PRDM).

PRDM Enhances the Activity of Heterologous Promoters in the Pineal Gland

The function of PRDM was tested in the context of other promoters to determine whether it could confer pineal specificity. This was first examined using a related gene, zebrafish *aanat-1* (*zfaanat-1*) that is expressed in the retina but not in the pineal gland (20). Constructs were prepared that contained the *zfaanat-1* promoter and EGFP reporter with or without PRDM (AANAT1-EGFP-PRDM and AANAT1-EGFP, Fig. 2A). AANAT1-EGFP injection resulted in 25% EGFP-expressing embryos, all of which exhibited low levels of ectopic expression (Fig. 2A). In contrast, injection of AANAT1-EGFP-PRDM led to pineal expression in 56% of the EGFP-positive embryos, half of which exhibited pineal-restricted expression; ectopic expression was found in 72% of EGFP-positive embryos (Fig. 2A). The apparent absence of retinal EGFP expression is related to the late differentiation of the retina or to the absence of elements in the cloned *zfaanat*-1 promoter that are essential for retinal expression. Nonetheless, these results demonstrate the ability of PRDM to confer *zfaanat-1* promoter activity in the pineal gland. It should be noted, however, that the percentage of embryos exhibiting ectopic expression (72%) was not affected by the presence of PRDM. Moreover, ectopic expression was significantly ($P < 0.001$) higher with AANAT1-EGFP-PRDM (72%) than with AANAT2-EGFP-PRDM (15%), suggesting that the *zfaanat-2* promoter contains negative regulatory elements that cooperate with PRDM to prevent extrapineal expression; and that these elements are not present in the *zfaanat-1* promoter.

The difference in the interaction of the PRDM with the two *aanat* promoters was confirmed in experiments in which AANAT1-EGFP-PRDM and AANAT2-DsRed-PRDM were coinjected (Fig. 2B); 77% of the EGFP-positive embryos exhibited ectopic expression, whereas among the DsRed-positive embryos only 10% exhibited ectopic expression; 39% and 90% of EGFP- and DsRed -positive embryos, respectively, exhibited pineal expression, resembling the results obtained by single-injection experiments. More importantly, individual embryos with pineal-specific expression also exhibited ectopic EGFP expression but not ectopic DsRed expression. Thus, replacing the *zfaanat-2* promoter with *zfaanat-1* promoter increased ectopic expression in individual embryos, reinforcing the different interactions of these promoters with PRDM. These experiments indicate that the PRDM functions as a repressor of extrapineal expression in the context of the *zfaanat-2* promoter and as an enhancer of pineal expression with both *zfaanat-1* and *-2* promoters.

The cytomegalovirus (CMV) promoter was also used to study the influence of the PRDM. This promoter induced a significantly ($P < 0.001$) higher percentage of EGFP-positive embryos as compared with the *aanat* promoters: injection of pCS2-CMV-EGFP resulted in 71% EGFP-positive embryos, all of which displayed extensive ectopic EGFP expression (Fig. 2, A and D). Pineal EGFP expression was detected in only 4% of positive embryos, apparently reflecting the intrinsic properties of the CMV promoter. Injection of pCS2-CMV-EGFP-PRDM resulted in similarly high percentage (81%) of ectopically expressing EGFP embryos, 43% of which, however, displayed pineal expression

original 3.5 kb, their designated letters, and the sequence that they span (note the overlapping regions) are depicted in the *middle column*. Injected embryos exhibit ectopic expression (*white bars*), pineal-specific expression (*black bars*), or both (*gray bars*). The percentages of each expression pattern out of the total positive fluorescence embryos are depicted on the *bar graph*. The numbers of EGFP-positive embryos out of total injected embryos (# fluorescent/total) are shown to the *right* of the bars. The proportion of pineal-expressing embryos is significantly affected by the 3′-fragment with highest proportion obtained with fragment D and E ($\chi 2$ analysis, $P < 0.001$). The proportion of ectopically expressing embryos is significantly affected by the 3′-fragment with lowest proportion obtained with fragment D ($\chi 2$ analysis, $P < 0.001$). B, The effect of fragment D was tested along with an internal control by comicroinjection of AANAT2-DsRed-d and AANAT2-EGFP (schematically shown on *left*). The percentages of each expression pattern of the total positive embryos are presented on the *right bar graph* for each reporter: red pineal (*diagonal striped bars*) or ectopic (*dotted white bars*), driven by AANAT2-DsRed-d, and ectopic green (*white bars*), driven by AANAT2-EGFP; in these experiments no green signal was identified in the pineal gland. The numbers of positive embryos out of total injected embryos are shown to the *right* of the bars (# fluorescent/total). Fragment D was subsequently renamed PRDM. C–I, Representative zebrafish embryos during the third day of development exhibiting combinations of expression patterns—ectopic EGFP (*yellow arrowhead*), pineal EGFP (*yellow arrow*), and pineal DsRed (*black arrow*)—after injection with the constructs described above. C, A group of embryos exhibiting ectopic EGFP expression. D, Ectopic EGFP expression (lateral view of head region). E, Both pineal and ectopic EGFP expression (dorsal view of the head). F, Both pineal and ectopic EGFP expression (lateral view). G, Pineal-specific expression of EGFP (lateral view). H, Pineal-specific expression of EGFP (dorsal view). I, Pineal-specific expression of DsRed (lateral view). J, Pineal DsRed expression along with ectopic EGFP expression during the fifth day of development (lateral view).

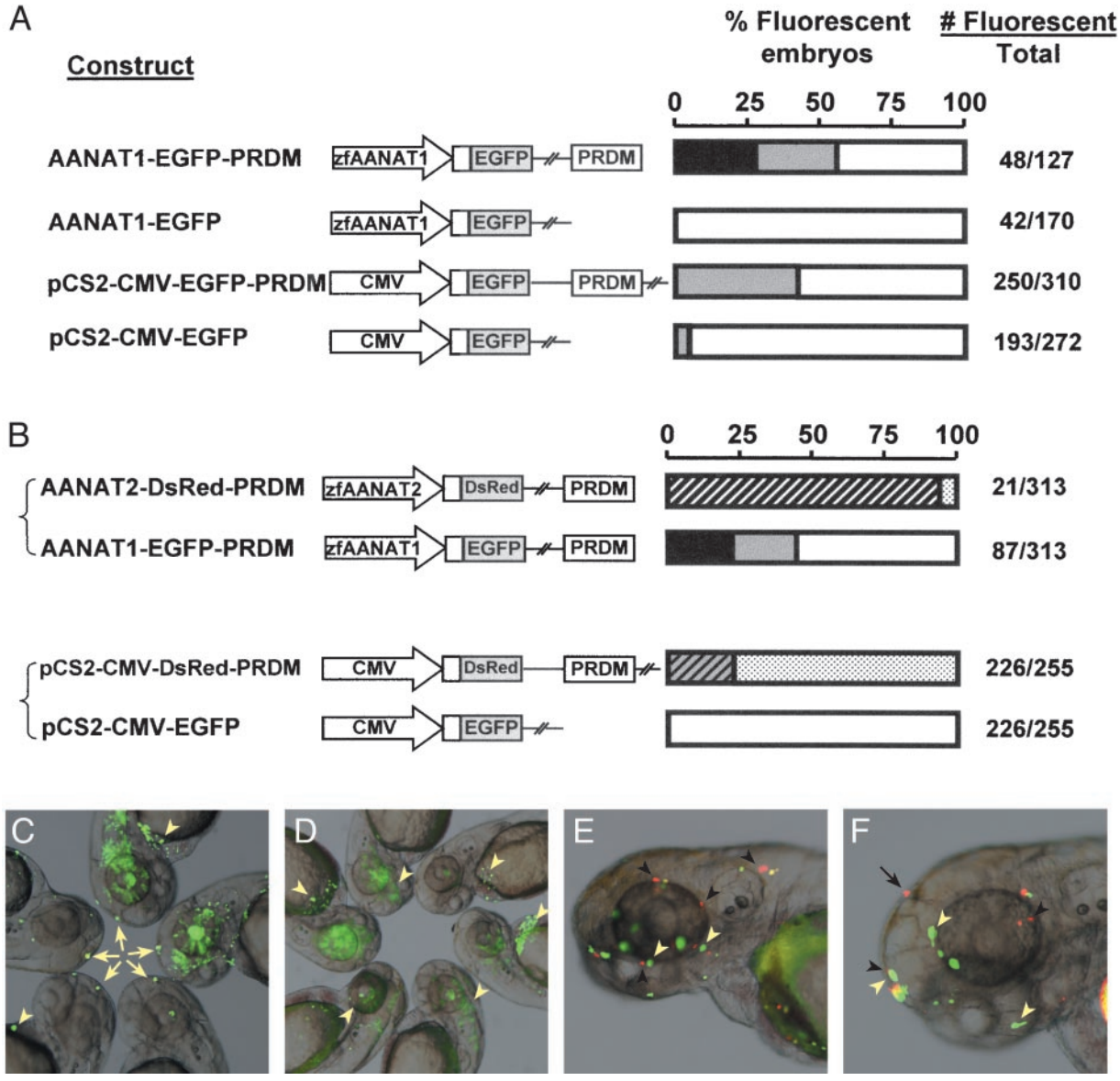

Fig. 2. PRDM Enhances Promoter Activity of Nonpineal Genes

A, PRDM was inserted into zfAANAT1-EGFP and CMV-EGFP promoter-reporter vectors. The names and structure of the injected constructs are shown on the *left*. The percentages of each expression pattern of the total positive embryos—ectopic (*white bars*), pineal (*black bars*), or both (*gray bars*)—are shown on the *right*. The numbers of positive embryos out of total injected embryos (# fluorescent/total) are shown to the *right* of the bars. The proportion of pineal-expressing embryos is significantly increased by PRDM (multiple logistic regression analysis, $P < 0.001$); there were no effects of the promoter or promoter-PRDM interactions on pineal expression. The proportion of ectopically expressing embryos is significantly affected by an interaction between PRDM and the promoter; the combination of PRDM and *aanat2* promoter reduces the level of ectopically expressing embryos 142-fold (multiple logistic regression analysis, $P < 0.001$). There were no effects of the promoter or PRDM alone on ectopic expression. B, The effect of PRDM on the activity of heterologous promoters was tested along with internal controls: comicroinjection of CMV-EGFP and CMV-DsRed-PRDM and comicroinjection of AANAT1-EGFP-PRDM and AANAT2-DsRed-PRDM (schematically shown on *left*). The percentages of each expression pattern of the total EGFP- or DsRed-positive embryos are depicted in the *bar graphs*. EGFP: pineal-specific (*black bars*), ectopic (*white bars*), and both (*gray*). DsRed: pineal-specific (*black and white diagonal striped bars*), ectopic (*dotted white bars*), and both (*black and gray diagonal striped bars*). The numbers of EGFP- or DsRed-positive embryos out of total injected embryos (# fluorescent/total) are shown to the *right* of the bars. C–F, Representative zebrafish embryos during the third day of development exhibiting combinations of transient expression patterns—ectopic EGFP (*yellow arrowhead*), pineal EGFP (*yellow arrow*), ectopic DsRed (*black arrowhead*), and pineal DsRed (*black arrow*)—after injection with CMV-EGFP-PRDM (C), CMV-EGFP (D), and coinjection of CMV-EGFP and CMV-DsRed-PRDM (E and F). C, A group of embryos exhibiting both pineal and ectopic EGFP expression. D, A group of embryos exhibiting ectopic EGFP expression. E, Ectopic expression of DsRed and EGFP (lateral view of head). F, Pineal DsRed expression along with ectopic DsRed and EGFP expression (lateral view of head).

(Fig. 2, A and C). These results indicate that the PRDM enhances pineal expression of the potent CMV promoter but did not confer exclusive pineal expression.

These results were confirmed in coinjection studies in which pCS2-CMV-DsRed-PRDM and pCS2-CMV-EGFP were used (Fig. 2B). Most (89%) of the injected embryos displayed both DsRed and EGFP ectopic expression (Fig. 2E). A quarter of the positive embryos exhibited pineal DsRed expression as well (Fig. 2F), whereas essentially no pineal EGFP expression was identified, confirming the results obtained by single-construct injection.

These results from studies with the heterologous promoters suggest that PRDM can enhance pineal expression of genes other than *zfaanat-2*, but cannot, by itself, repress extrapineal expression in the context of these promoters. Overall, the above data indicate that the effect of the PRDM is influenced by the promoter present. That is, it increases ($P < 0.001$) the proportion of pineal-expressing embryos in the context of all three promoters but decreases ($P < 0.001$) the proportion of ectopic expression only when in combination with the *zfaanat-2* promoter.

Identification of Three Photoreceptor Conserved Elements (PCEs) and an E Box in the PRDM

Manual and computer analysis of the PRDM sequence revealed that it contains several putative transcription factor binding sites. Of special interest are three pentamers (TAATC) centered at positions 1278, 1343, and 1414 (Fig. 3); these are identical to the pineal gland-regulatory element (PIRE, Table 1 and Ref. 24) and essentially the same as the PCE (Table 1), first identified in photoreceptor-specific genes (25). Both PCE and PIRE bind the mammalian cone rod homeobox protein [CRX (24, 26)], a transcription factor that influences photoreceptor- and pineal-selective gene expression and photoreceptor differentiation (27, 28), and are therefore functionally the same element. The PCE/PIRE sequence is similar to the rhodopsin core sequence, which was shown to determine photoreceptor-specific expression of the *Drosophila rhodopsin* (Table 1 and Ref. 29). Selection of the term PCE here reflects the broad and conserved function that this sequence seems to play in determining photoreceptor specificity of an array of genes.

Another transcription factor binding site of special interest is a perfect E-box (CACGTG) at position 1261–1266 (Fig. 3). This short hexameric DNA core sequence can mediate different transcriptional outputs depending upon the context, *i.e*. the features of its surrounding sequence and cellular environment (17, 30). Early studies on this regulatory element revealed a role in cell-specific expression during developmental myogenesis (31). More recently, the E-box has been found to be an essential element of the transcription-translation feedback loop that constitutes the circadian clock and is responsible for turning on clock-controlled genes (16, 32), including chicken *aanat* (14).

Intact PCEs Are Required for PRDM Function

The function of the PRDM PCEs was examined using a construct (AANAT2-EGFP-PRDM-MP1–3) in which all three PCEs were mutated. Injection of this construct failed to generate pineal expression and increased the occurrence of ectopic expression: all EGFP-positive embryos (26% of total injected) displayed ectopic expression (Fig. 4A). The influence of each PCE was examined using constructs in which each was mutated (AANAT2-EGFP-PRDM-MP1, -MP2, and -MP3). Mutating any one PCE reduced ($P < 0.001$) pineal expression and increased ($P < 0.001$) ectopic expression as compared with the wild-type PRDM (Fig. 4A). These results suggest that each of the three PCEs contributes to the dual PRDM function of enhancing pineal expression and inhibiting extrapineal expression.

There is no direct evidence for the involvement of CRX in determining pineal specificity in fish. Nevertheless, a region containing PCEs was shown to be involved in determining pineal-specific expression of the zebrafish exo-rhodopsin gene (33). Moreover, zebrafish orthodenticle homeobox 5 (zOTX-5), an ortholog of the mammalian Crx genes (34), has been shown to augment the expression of clock-controlled genes, including *zfaanat-2*, in the pineal gland. Knockdown of zOTX-5 has reduced the level of these rhythmic genes (21), and microinjection of zOTX-5 mRNA in

Fig. 3. Nucleotide Sequence of the PRDM

Numbers correspond to the 3.5-kb downstream sequence (accession no. AY380805). The E-box and PCEs are *underlined* and labeled accordingly.

Table 1. PCE and PCE-Like *cis*-Acting Elements

Element	Activity Site	Sequence	Reference No.
Rh3 RCSI[a]	*Drosophila* visual system	C**TAATC**CAATT	29
Rh4 RCSI	*Drosophila* visual system	C**TAATT**GAATT	29
PCE	Mouse pineal and retina	sC**TAATT**Gr	25
PIRE	Rat pineal	**TAATy**	24
PRDM-PCE	Zebrafish pineal	**TAATC**	Current study

The consensus sequence among these elements is shown in *boldface,* emphasizing their possible common role.
[a] RCS, Rhodopsin core sequence.

TG(AANAT2:EGFP) line has induced, among other developmental abnormalities, ectopic expression of EGFP (data not shown). Taken together, it is possible that the three PCEs within the PRDM mediate CRX/OTX enhancement of pineal expression. In addition, the results of our experiments indicate that these same elements have a second function: to inhibit extrapineal expression; this might involve unknown binding protein(s).

An Intact E Box Is Required for PRDM Function

In the context of a clock-controlled gene and a clock-containing organ, an E-box would be predicted to mediate rhythmic expression (17). However, the identification of an E-box in PRDM raised the question of whether this element is involved in restricting the expression of *zfaanat-2* to the pineal gland. To clarify this question, a construct containing a mutated PRDM E-box (AANAT2-EGFP-PRDM-ME, Fig. 4A), was injected. This mutation reduced the pineal gland-specific and increased ectopic expression as compared with wild-type PRDM ($P < 0.001$); among the EGFP-expressing embryos, only 10% showed pineal-specific expression, 16% had both pineal and ectopic expression, and, 74% showed ectopic expression. These results indicate that the PRDM E-box is essential for the dual action of PRDM.

This was confirmed in coinjection experiments using AANAT2-DsRed-PRDM and AANAT2-EGFP-PRDM-ME constructs. Fifty percent of the injected embryos showed EGFP or DsRed expression. Most of the EGFP expression was ectopic (95%), whereas DsRed expression was mostly pineal specific (90%, Fig. 4B). Every embryo with pineal-specific DsRed expression also exhibited ectopic EGFP expression. Hence, two-point mutations in the E-box are sufficient to shift expression from pineal specific to ectopic in each individual embryo, reinforcing the importance of this E-box in determining pineal specificity. These results strongly suggest a novel function for an E-box in the establishment of pineal-specific expression by both enhancing pineal expression and suppressing extrapineal expression.

The ability of the E-box alone to drive pineal expression was tested by introducing a perfect E-box into an otherwise inactive fragment, fragment F (Fig. 1A). The resulting construct (AANAT2- EGFP-MF, Fig. 4A), was injected, and 20% of the injected embryos showed EGFP expression, all of which was ectopic; pineal EGFP expression was not detected (Fig. 4A). This finding was confirmed in coinjection experiments using AANAT2-EGFP-MF and AANAT2-DsRed-PRDM as the internal reference (Fig. 4B). Nearly half of the injected embryos showed EGFP or DsRed expression. Among the EGFP-positive embryos, 96% were ectopic, whereas in 90% of the DsRed-positive embryos, expression was restricted to the pineal gland. More importantly, 80% of the pineal gland-specific DsRed-expressing embryos also showed ectopic EGFP expression. These experiments demonstrate that the E-box alone is not sufficient to drive pineal-specific expression and strengthens the importance of sequence context (17, 30, 35)—in this case the PRDM. This context consists of the three PCEs and may also include nucleotides flanking the E-box or other regulatory sequences within the PRDM.

DISCUSSION

The regulatory mechanisms responsible for temporal and tissue-specific gene expression constitute an essential component in the developmental control specifying the identity and function of the pineal gland. This photoneuroendocrine organ appears to share a common developmental and evolutionary origin with the photoreceptor cells of the retina. Both express similar, but not identical, sets of genes dedicated to photoreception, phototransduction, and melatonin synthesis. The overlaps of transcribed genes vary among vertebrate classes and are most prominent in teleost fish where duplicate genes with apparently parallel functions are usually expressed in either the retina or the pineal gland (20). Accordingly, it may be hypothesized that gene expression in these tissues is determined by distinct, but very closely related, regulatory mechanisms, which are presently unclear. For example, rhodopsin expression in the mammalian retina is driven by the synergistic action of CRX, common to both tissues, and neural retina leucine zipper, a retina-specific factor (26). Likewise, exorhodopsin expression in the zebrafish pineal gland is driven by two *cis*-acting elements: PCE, which probably mediates CRX/OTX action, and pineal expression-promoting element, which mediates the action of an as-yet-unknown factor (33).

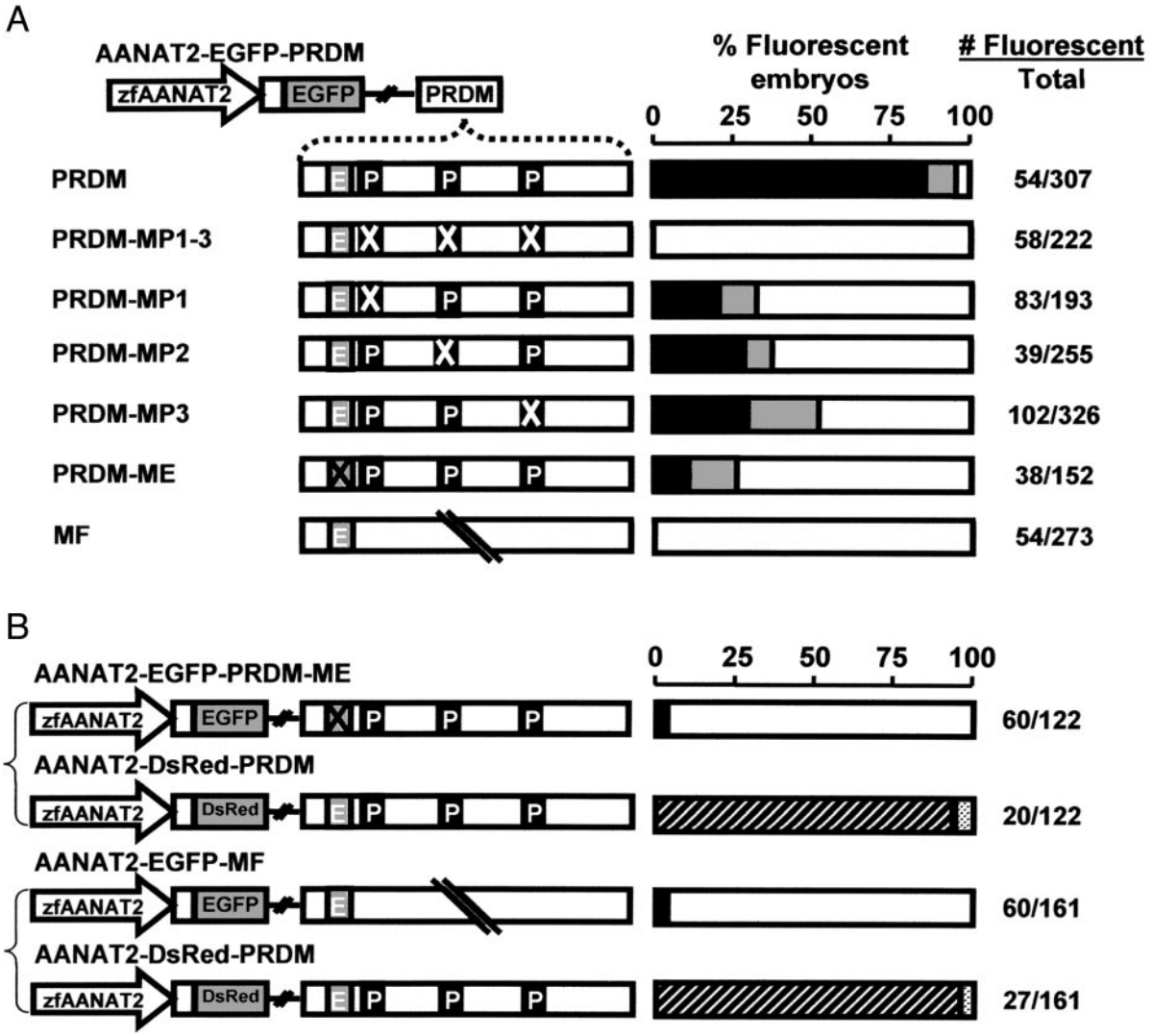

Fig. 4. Mutation Analysis of PRDM Elements

A, The perfect E-box (E) or PCEs (P) sites within the PRDM were mutated (X; see *Materials and Methods*), and the resulting constructs were tested. The names of the mutated constructs and the relative position of the disrupted elements (E and P) within the PRDM are shown on the *left*. The percentages of each expression pattern out of the total fluorescent embryos are depicted in the *bar graph*: ectopic (*white bars*), pineal (*black bars*), or both (*gray bars*). The proportion of pineal-expressing embryos was significantly reduced by PCE and E-box mutations ($\chi 2$ analysis, $P < 0.001$). The proportion of ectopically expressing embryos was significantly increased by PCE and E-box mutations ($\chi 2$ analysis, $P < 0.001$). B, The effects of E-box mutation, AANAT2-EGFP-PRDM-ME, and E-box knock-in, AANAT2-EGFP-MF, were tested along with an internal reference by their comicroinjection with AANAT2-DsRed-PRDM. The names and structure of injected constructs are shown on the *left*. The percentages of each expression pattern of the total EGFP- or DsRed-positive embryos are depicted in the *bar graph*: red pineal (*diagonal striped bars*) or ectopic (*dotted white bars*), driven by the internal control construct, AANAT2-DsRed-PRDM; green pineal (*black bars*) or ectopic (*white bars*) are driven by mutant constructs (E-box mutation and E-box knock-in). The numbers of positive embryos out of total injected embryos (# fluorescent/total) are shown to the *right* of the bars.

The current study extends our understanding of how tissue-specific expression of the *zfaanat-2* gene is regulated and may add general knowledge about pineal specificity. It appears that the expression of *zfaanat-2* in the pineal gland is controlled by a bipartite regulatory system composed of two regions separated by 12 kb. One is the upstream promoter, which is sufficient to drive ectopic gene expression, and the other is the 257-bp PRDM, which is located more than 6 kb downstream to the transcribed region. Acting together, these two regions enhance pineal gland expression while suppressing it elsewhere. This bipartite organization is also seen in rat *aanat*, in which tissue specificity is dependent upon both the 5′-flanking region and a downstream intronic region (36, 37). In addition, the PRDM appears to be able to drive pineal expression of extrapineal promoters but does not have an effect on their extrapineal activity (Table 2).

The results of the current study demonstrate that the combination of an E-box and three PCEs is required for optimal function of the PRDM in restricting *zfaanat-2* expression to the pineal gland. In the context of this clock-controlled gene and clock-containing organ, an E-box would be expected to mediate the rhythmic gene expression. This function is assumed to be mediated by two 5′-E-box elements (18). As discussed above, the E-box is known to play a role, among many others, in myocyte differentiation (31, 38). The demonstrated function of the PRDM E-box expands this role to determination of pineal specificity. The second required element, PCE, may serve as binding sites for homeobox proteins of the CRX/OTX family. As mentioned above, zOTX-5 has been implicated in enhancing the expression of clock-controlled genes in the pineal gland. The identification of functional PCEs within the PRDM is consistent with a direct action of zOTX-5 on regulatory regions of clock-controlled genes in the zebrafish pineal gland (21).

The combination of E-box and PCE in determining tissue specificity may not be unique to zebrafish *aanat-2*: it exists in the rat *aanat* intronic region, which, as mentioned above, is required for tissue specificity. Similarly, analysis of the Fugu genome revealed a region, downstream of the AANAT-2 gene that, like the zebrafish PRDM, contains an E-box and three PCEs with similar orientation and distances (bases 60,502–60,and 738 of scaffold 180, http://www.ensembl.org/Fugu_rubripes). The function of this genomic sequence as well as the E-box and PCEs in the rat *aanat* intronic fragment requires further functional investigation; however, the presence of this E-box/PCE downstream cluster in *aanat* of three different species points to the hypothetical possibility that this cluster may contribute in a more general manner to pineal-specific expression of AANAT and other genes in vertebrates. The recognized role of the BMAL/CLOCK heterodimer and zOTX-5 in the regulation of clock-controlled genes raises an attractive possibility that the combination of E-box and PCEs mediates both rhythmic expression and specificity in clock-containing brain structures, thus linking temporal and spatial expression of genes in the brain.

Table 2. Different Interaction of PRDM with Homologous and Heterologous Promoters

Promoter	Pineal Expression		Ectopic Expression	
	PRDM	–	PRDM	–
zfaanat-2	↑	↓	↓	↑
zfaanat-1	↑	↓	nc	nc
CMV	↑	↓	nc	nc

Arrow direction indicates increased or decreased level of expression. $P < 0.001$ by multiple logistic regression analysis; nc, no change.

The results presented in this paper, together with the published data discussed above, suggest that pineal-restricted expression, at least of the *zfaanat-2*, is achieved by the combination of pineal-enhancing and extrapineal-repressive mechanisms. These mechanisms involve several interacting PCEs, a perfect E-box, and probably other elements within the PRDM. The identification of proteins that are recruited onto the PRDM in the pineal gland and nonpineal tissues requires further analyses. The zebrafish will constitute a valuable model system in that regard because it offers a growing number of available molecular genetic techniques including mutagenesis-, overexpression-, and knockdown-based screening of the publicly available gene sequences. Furthermore, the TG(AANAT2:EGFP) and future transgenic lines may serve as a model for analyzing the effect of clock-controlled genes and other transcription factors on pineal-specific and rhythmic gene expression. Such further studies, together with targeted data mining of emerging selected genomes, will lead to a better understanding of mechanisms that turn on high expression in the pineal gland while turning it off elsewhere in the organism.

MATERIALS AND METHODS

Fish Maintenance

Zebrafish adults and larvae were raised and maintained under optimal conditions (39). Embryos were generated by natural mating and were raised at 28 C in egg water containing methylene blue (0.3 ppm). To prevent pigmentation, 0.2 mM phenylthiourea was added to the water during the second day of development.

DNA Constructs

AANAT2-EGFP (Fig. 1A). The *zfaanat-2* promoter was subcloned into pEGFP-1 (CLONTECH, Palo Alto, CA) upstream of the EGFP reporter gene. A fragment containing 1.65 kb of 5′-flanking region and the 123-bp 5′-untranslated region of the *zfaanat-2* (GenBank accession no. AF494081) was PCR amplified as previously described (18), digested with *Bam*HI and *Sal*I and ligated into *Bam*HI/*Sal*I-cut pEGFP-1.

AANAT2-EGFP−3.5 kb (Fig. 1A). A 3.5-kb *Aat*II/*Sac*II fragment derived from the *zfaanat-2* 3′-flanking region, was placed in AANAT2-EGFP, downstream to the EGFP-coding region. This fragment was first cloned into *Aat*II/*Sac*II-cut pGEM-T Easy (Promega Corp., Madison, WI), yielding pGEM-3.5. The majority of the insert (3.4 kb, GenBank accession no. AY380805) was excised with *Sal*I and *Stu*I located near the 5′- and 3′-ends of the 3.5-kb insert, respectively, and was subcloned into *Eco47* III/ *Xho*I-cut AANAT2-EGFP.

AANAT2-EGFP+3.5 kb-derived fragments (Fig. 1A). Overlapping fragments derived from the 3.5-kb downstream region were PCR amplified using pGEM-3.5 as a template and sets of primers containing *Xho*I and *Eco*RI restriction sites. PCR products were double digested with *Xho*I and *Eco*RI and ligated into *Xho*I/*Eco*RI-cut AANAT2-EGFP, yielding constructs AANAT2-EGFP-a trough AANAT2-EGFP-h (Fig. 1A). Smaller fragments within fragment D were PCR amplified and subcloned into AANAT2-EGFP to generate AANAT2-EGFP-d1 and AANAT2-EGFP-d2 (Fig. 1A).

AANAT2-DsRed-d (Fig. 1B). The coding region of EGFP in AANAT2-EGFP-d was replaced by the coding region of the red fluorescent protein (DsRed) reporter gene. DsRed coding region was PCR amplified using pDsRed1-N1 vector (CLONTECH) as template and set of primers containing restriction sites. The PCR product was digested with *Bam*HI and *Not*I and ligated into *Bam*HI/*Not*I-cut AANAT2-EGFP-d, replacing EGFP, to produce AANAT2-DsRed-d (Fig. 1B).

Fragment D was subsequently renamed pineal restrictive downstream module (PRDM); accordingly, construct AANAT2-EGFP-d and AANAT2-DsRed-d were renamed AANAT2-EGFP-PRDM and AANAT2-DsRed-PRDM, respectively.

pCS2-CMV-EGFP (Fig. 2A). This construct, in which EGFP expression is driven by the CMV promoter, was kindly provided by Joe Breen, National Institute of Allergy and Infectious Diseases (Bethesda, MD).

pCS2-CMV-EGFP-PRDM (Fig. 2A). Fragment D (PRDM) was subcloned into pCS2-CMV-EGFP, downstream to the EGFP coding region. PRDM was PCR amplified using pGEM-3.5 as template and a set of primers containing *Apa*I and *Sac*II restriction sites. The product was digested with *Sac*II and *Apa*I and ligated into *Sac*II /*Apa*I-cut pCS2-CMV-EGFP.

pCS2-CMV-DsRed-PRDM (Fig. 2B). The coding region of EGFP in pCS2-CMV-EGFP-PRDM was replaced by the coding region of DsRed reporter gene. PCR amplified DsRed coding region (obtained as described above) was digested with *Bam*HI and *Xba*I and ligated into *Bam*HI/*Xba*I-cut pCS2-CMV-EGFP-PRDM, replacing the EGFP coding region.

AANAT1-EGFP and AANAT1-EGFP-PRDM (Fig. 2A). *zfaanat-1* promoter was placed upstream of EGFP in two constructs. A fragment containing 784 bp of 5′-flanking region and 92 bp 5′-untranslated region of *zfaanat-1* was PCR amplified from zebrafish genomic DNA based on cDNA (GenBank accession no. AY349158) and genomic (chromosome fragment NA10279, http://www.ensembl.org/Danio_rerio) sequences, using a pair of specific primers containing *Sal*I and *Bam*HI restriction sites. The product was digested with *Sal*I and *Bam*HI and ligated into *Sal*I/*Bam*HI-cut AANAT2-EGFP and AANAT2-EGFP-PRDM constructs, replacing *zfaanat-2* promoter.

AANAT2-EGFP-PRDM + PCE Mutation (Fig. 4A). Initially, each of the three PCEs within the PRDM was mutated. For Each PCE two complementary primers containing the desired mutation were used to introduce the mutations into AANAT2-EGFP-PRDM construct using QuikChange Site-directed mutagenesis kit (Stratagene, La Jolla, CA) as instructed by manufacturer. Three sets of complementary primers were used to mutate TAATC (PCE1, Fig. 3) to AGATC, GATTA (PCE2) to GTATA, and TAATC (PCE3) to ATATC. Consequently, novel restriction sites, *Bgl*II, *Eco*RV, and *BsT*Z17I, were introduced into the sequence in place of PCE1, PCE2, and PCE3, respectively. Mutated colonies were selected based on their digestion pattern with the above enzymes, and the presence of mutation was confirmed by sequencing. These constructs were named AANAT2-EGFP-PRDM-MP1, -MP2, and -MP3 (Fig. 4A). Next, AANAT2-EGFP-PRDM-MP1 was sequentially mutated at PCE2 and PCE3 to get a triple PCE mutation construct, AANAT2-EGFP-PRDM-MP1–3.

AANAT2-EGFP-PRDM-ME (Fig. 4A). The E-box within PRDM was mutated. Two complementary primers were used to introduce the desired mutations (CACGTG to CTCGAG) into AANAT2-EGFP-PRDM using a similar procedure as used for the PCE mutations. Colonies with the desired mutation were selected on the basis of *Pml*I and *Xho*I digestion pattern and sequencing.

AANAT2-EGFP-MF (Fig. 4A). A perfect E-box was introduced into fragment F. Two complementary primers were employed to introduce a mutation into AANAT2-EGFP-f as described above. As a result, guanidine located at position 1779 (GenBank accession no. AY380805) was replaced by an adenosine, and the original sequence, CGCGTG, was converted to a perfect E-box, CACGTG. Colonies with the desired mutation were selected based on *Pml*I digestion pattern and sequencing.

Transient Expression Assay

Transient expression assays of the DNA constructs listed above were performed by microinjection of zebrafish embryos as described previously (18). DNA constructs were purified using a plasmid isolation kit (QIAGEN, Valencia, CA) and diluted to a final concentration of 100 ng/μl injection solution (0.1 M KCl/0.05% phenol red). In coinjection experiments, constructs were dissolved together to a final concentration of 100 ng/μl injection solution each. Approximately 2 nl were injected into the cytoplasm of one- or two-cell-stage wild-type zebrafish zygotes using micromanipulator and PV830 Pneumatic Pico Pump (World Precision Instruments, Sarasota, FL). Embryos (100–400) were injected using three to four different needles in each experiment and were incubated in a 10-cm plastic dish at 28 C.

Examination of Embyros and Statistical Analysis

Injected embryos were maintained under 12-h light, 12-h dark cycles and repeatedly examined and graded during the light phase on d 2–5 post fertilization. Green fluorescence in live embryos was detected under a stereo dissecting microscope (MZ9, Leica Corp., Deerfield, IL) equipped with a fluorescent module having a EGFP filter set for excitation (460–500 nm) and emission (>510 nm). Codetection of green and red fluorescence was done using an Olympus dissecting microscope SZX12 equipped with filters for excitation (460–490 nm) and emission (510–550 nm) of EGFP and excitation (460–560 nm) and emission (>590 nm) of DsRed. Due to the limited stability of the injected DNA and the increased stability of the fluorescent proteins, signals detected after d 3 might primarily reflect accumulation of the stable protein, which could mask day-night difference (40). Embryos were sorted on the basis of their expression pattern: an individual was considered “pineal specific” if fluorescence was detected only in the pineal gland; “ectopic” if at least one extrapineal fluorescent signal was detected in the absence of a pineal signal; and, “both” if fluorescent signals were detected in the pineal gland and in extrapineal sites. At the first observation (d 2 post fertilization), embryos with similar expression pattern were housed together in separate 10-cm dishes (<60 embryos per dish) for additional daily observation and validation until d 5.

The single construct injection data were subjected to statistical analyses as detailed here. The significance of the effects of the promoter (CMV, AANAT1, and AANAT2), the downstream fragment (a–h), and the mutations and interactions of these variables on the proportion of embryos with pineal *vs.* ectopic expression was determined by stepwise logistic regression (41). Specific comparisons were performed using χ^2 analysis (41). Conclusions were confirmed by double-injection experiments. The proportion of each expression pattern is presented in the *bar graphs* of Figs. 1, 2, and 4 as percentage of the total fluorescent embryos (% fluorescent embryos). The number of embryos exhibiting a florescent signal and the total number of injected embryos is given as “# fluorescent/total.” The probability for observed differences is given in the text.

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